Interruption of spinal cord microglial signaling by alpha-2 agonist dexmedetomidine in a murine model of delayed paraplegia

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Background: **Despite investigation into preventable pharmacologic adjuncts, paraplegia continues to complicate thoracoabdominal aortic interventions. The alpha 2a adrenergic receptor agonist, dexmedetomidine, has been shown to preserve neurologic function and neuronal viability in a murine model of spinal cord ischemia reperfusion, although the mechanism remains elusive. We hypothesize that dexmedetomidine will blunt postischemic inflammation in vivo following thoracic aortic occlusion with in vitro demonstration of microglial inhibition following lipopolysaccharide (LPS) stimulation.**

Methods: **Adult male C57BL/6 mice underwent 4 minutes of aortic occlusion. Mice received 25 μg/kg intraperitoneal dexmedetomidine (n = 8) or 0.9% normal saline (n = 7) at reperfusion and 12-hour intervals postoperatively until 48 hours. Additionally, sham mice (n = 3), which had aortic arch exposed with no occlusion, were included for comparison. Functional scoring was done at 6 hours following surgery and 12-hour intervals until 60 hours when spinal cords were removed and examined for neuronal viability and cytokine production. Additional analysis of microglia activation was done in 12 hours following surgery. Age- and sex-matched mice had spinal cord removed for microglial isolation culture. Cells were grown to confluence and stimulated with toll-like receptor-4 agonist LPS 100 ng/mL in presence of dexmedetomidine or vehicle control for 24 hours. Microglia and media were then removed for analysis of protein expression.**

Results: **Dexmedetomidine treatment at reperfusion significantly preserved neurologic function with mice in treatment group having a Basso Score of 6.3 in comparison to 2.3 in ischemic control group. Treatment was associated with a significant reduction in microglia activation and in interleukin-6 production. Microglial cells in isolation when stimulated with LPS had an increased production of proinflammatory cytokines and markers of activation. Treatment with dexmedetomidine significantly attenuated microglial activation and proinflammatory cytokine production in vitro with a greater than twofold reduction in tumor necrosis factor-α.**

Conclusions: **Alpha 2a agonist, dexmedetomidine treatment at reperfusion preserved neurologic function and neuronal viability. Furthermore, dexmedetomidine treatment resulted in an attenuation of microglial activation and proinflammatory cytokine production both in vivo and in vitro following LPS stimulation. This finding lends insight into the mechanism of paralysis following thoracic aortic interventions and may guide future pharmacologic targets for attenuating spinal cord ischemia and reperfusion. (J Vasc Surg 2014;59:1090-7.)**

Clinical Relevance: **Despite the evolution of surgical techniques, paraplegia continues to complicate aortic interventions. We have previously shown that pre- and postoperative administration of dexmedetomidine, a commonly used sedation agent, provides significant neuroprotection in a murine model of spinal cord ischemia-reperfusion. Although dexmedetomidine has shown protection in multiple tissue beds, its mechanism of protection remains highly debated. In our model of spinal cord ischemia-reperfusion, dexmedetomidine exerts significant anti-inflammatory effects in vivo when given postoperatively and implicates dexmedetomidine's anti-inflammatory effects on the resident macrophages in the central nervous system.**

Delayed, progressive paraplegia remains a significant complication of thoracoabdominal aortic interventions.[1] Postischemic inflammatory responses are believed to be major contributors to secondary injury and the development of delayed paraplegia.[2] The role of resident macrophages in exacerbation of tissue injury following ischemia has been demonstrated in the central nervous system (CNS) as well as other tissue beds.[3,4] Pharmacologic adjuncts to attenuate the postischemic inflammatory sequelae have been a long sought after and a logical target for reducing the incidence of delayed paraplegia, yet no current options are available.

Microglia, the resident macrophages in the CNS, produce both beneficial and toxic effects.[5] Spinal cord injury induces a rapid change in microglial morphology, gene expression, and functional behavior.[6] Once activated, microglia can produce a host of neurotoxic mediators such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and nitric oxide.[5] Stimulation of microglial with bacterial

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Table. Basso Mouse Scale for locomotion

Score	*Operational definition (Basso et al[13])*
0	No ankle movement
1	Slight ankle movement
2	Extensive ankle movement
3	Plantar placing of the paw with or without weight support, or occasional, frequent -OR- Consistent dorsal stepping but no plantar stepping
4	Occasional plantar stepping
5	Frequent or consistent plantar stepping, no coordination -OR- Frequent or consistent plantar stepping, some coordination, paws rotated at initial contact and lift off
6	Frequent or consistent plantar stepping, some coordination, paws parallel at initial contact -OR- Frequent or consistent plantar stepping, mostly coordinated, paws rotated at initial contact and lift off
7	Frequent or consistent plantar stepping, mostly coordinated, paws parallel (P) at initial contact and rotated (R) at lift off (P/R) -OR- Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), and severe trunk instability
8	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), and mild trunk instability -OR- Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), and normal trunk stability and tail down or up and down
9	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), and normal trunk stability and tail always up

lipopolysaccharide (LPS) can readily induce microglia to transform into a neurotoxic phenotype seen acutely following ischemia.[7-9]

Dexmedetomidine is a highly selective α-2a adrenoreceptor agonist with well-documented neuroprotective effects.[10,11] Many have postulated that the protective effects are secondary to its anti-inflammatory characteristics, however, these remain ill-defined. We hypothesize that dexmedetomidine will blunt postischemic inflammation in vivo following thoracic aortic occlusion with in vitro demonstration of an inhibition of microglial activation and cytokine production following LPS stimulation.

METHODS

Animal procedures. All experiments were approved by the Animal Care and Use Committee at the University of Colorado, and this investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (www.nap.edu/catalog/5140.html).

Ischemia-reperfusion (IR) surgery. Eighteen C57BL/6 male mice 12 to 20 weeks were obtained from Jackson Laboratories (Sacramento, Calif). Using 2% isoflurane exposure of the thoracic aortic arch and subclavian artery were obtained through a cervicothoracic approach.[12] The aortic arch and subclavian artery were then occluded with vascular

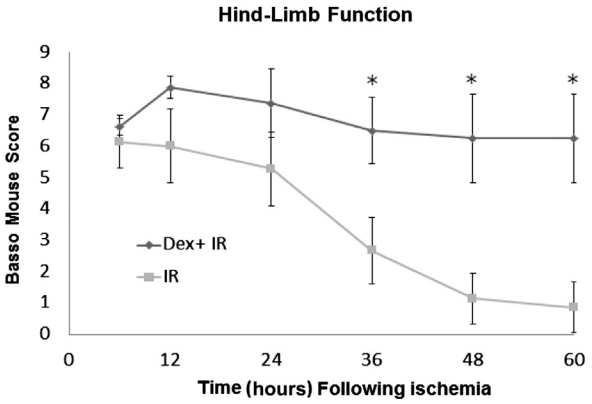

Fig 1. Hindlimb function, as characterized by the Basso Mouse Score, demonstrated a progressive decline in function of ischemia-reperfusion (*IR*) controls. Mice treated with dexmedetomidine (*Dex*) had a similar initial decline; however, their function stabilized and was significantly higher (*$P < .05$) than IR controls from 36 to 60 hours of reperfusion.

clamps placed under direct visualization. Occlusion was confirmed by a laser Doppler monitor (Moor Instruments, Wilmington, Del) over the left femoral artery and maintained for 4 minutes. A 90% decrease in distal aortic flow was present in all mice. Intraoperative body temperature was maintained at 36.5 ± 0.5°C using rectal temperature probe and automatic temperature adjusting bed (Vestavia Scientific, Birmingham, Ala).

Animals in the treatment group (n = 8) received intraperitoneal 25 μg/kg dexmedetomidine, based on previous studies,[4] 30 minutes following aortic occlusion and at 12-hour intervals postoperatively. IR controls (n = 7) received equivalent amounts of 0.9% at the points as specified above. Sham mice (n = 3) had the aortic arch exposed through the same procedure but no aortic cross clamping. All animals were sacrificed at 60 hours after surgery, and their spinal cords were harvested for analysis.

Functional assessment. Neurologic function was quantified at 6, 12, 24, 36, 48, and 60 hours using Basso Mouse Scale for locomotion (Table)[13] with values ranging from 0 for complete lower extremity paralysis to 9 for normal function.

Tissue procession. Following 60 hours of reperfusion, all animals were sacrificed and vertebral columns were removed en bloc from T8-L3. The spinal cord was removed from the vertebral column by injection of phosphate-buffered saline (PBS; pH 7.4) into the spinal column and transferred to 4% formalin for 24 hours. Following fixation, tissue was embedded in paraffin and stained with hematoxylin and eosin.

Spinal cords intended for cytokine analysis were removed at 60 hours as above then flash-frozen using 2-methylbutane (Fisher Scientific, Fair Lawn, NJ) and dry ice and stored at −80°C. Samples were then thawed to 4°C, weighed and homogenized using 10 μL/mg of ethylenediaminetetra-acetic acid-free, complete lysis-M buffer (Roche Diagnostics Corporation, Indianapolis,

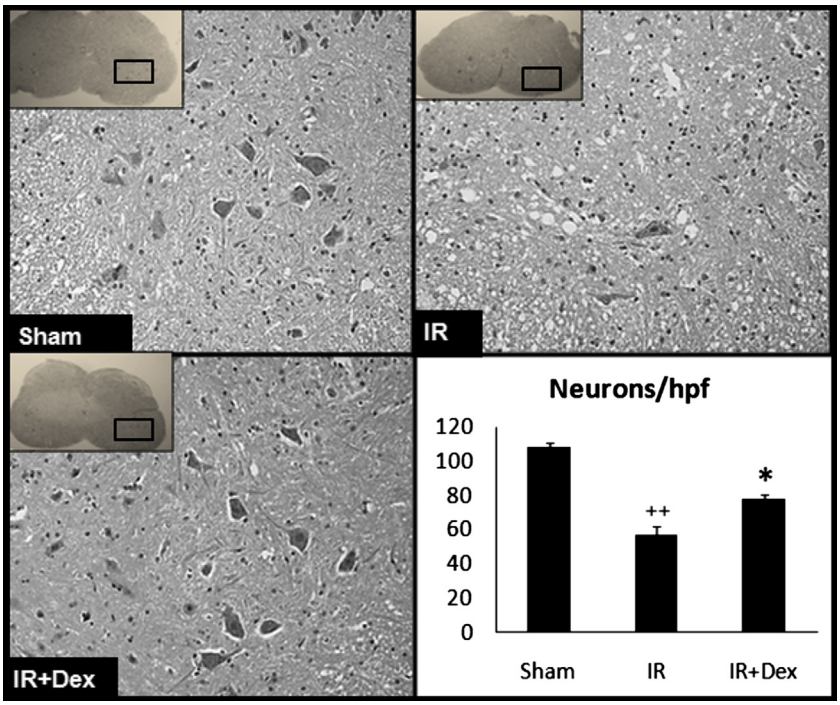

Fig 2. Representative slices of spinal cords following sham or IR surgery. Images are shown at original magnification ×40 with images in the box at original magnification. Mice treated with dexmedetomidine (*IR+Dex*) had more normal appearing anterior horn motor neurons with less gray-white matter disruption and fewer inflammatory infiltrates compared with ischemia-reperfusion (*IR*) controls. Normal appearing anterior horn neurons were quantified by a blinded observer. All mice undergoing IR surgery had significantly less anterior horn neurons ($++P < .05$) compared with compared with sham mice. Comparison of mice that underwent IR surgery revealed that mice treated with dexmedetomidine (*IR+Dex*) had a significantly higher quantity of normal anterior horn neurons ($*P < .05$).

Ind). Protein quantification was performed for each sample using NanoDrop ND-100 Spectrophotometer (Thermo Scientific, Wilmington, Del).

Histologic analysis and neuronal quantification. Sections and examined for cyto-architectural characteristics following hematoxylin and eosin. Neurons within the anterior horn that contained prominent nucleoli and loose chromatin were considered normal. Neuronal viability was quantified by a blinded observer and recorded as neurons per microscopic field.

Immunofluorescence. Twelve hours following IR surgery, six mice, three treatment, and three IR controls had spinal cords removed and fixed as described above. Following fixation, samples were placed in Tissue Freezing Medium (Electron Microscopy Sciences, Hatfield, Pa) at −80°C. Cryosections (10-μm thick) were cut and collected on poly-l-lysine-coated slides. Slides were treated with a mixture of 30% acetone and 70% methanol for 10 minutes then blocked with 10% normal donkey serum for 45 minutes. Primary incubation occurred overnight with a polyclonal rabbit anti-mouse ED-1 (Santa Cruz Biotechnology, Santa Cruz, Calif) at a 1:150 dilution and immune-matched IgG (5 μg/mL) containing 1% bovine serum albumin (BSA). Secondary incubation was perfumed for 1 hour with Cy3-conjugated matched IgG (Jackson Laboratories, Inc, West Grove, Pa) at 1:150 dilution with PBS containing 1% BSA. Bis-benzimide was used (4′,6-diamidino-2-phenylindole, imaged on the blue channel) to stain nuclei and wheat-germ agglutinin to stain cell membranes (labeled with Alexa 488 and imaged on the green channel). ED-1 was imaged using the red Cy3 channel. Microscopic observation and photography were performed by Leica DMRXA confocal microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany).

Microglial isolation. Male mice 12 to 20 weeks had vertebral column removed en bloc. Spinal cords were cut longitudinally and transferred to cold, Hibernate A (Brain Bits, LLC, Springfield, Ill) with glutamax 0.5 mM and 10% fetal bovine serum (Invitrogen, Grand Island, NY). Sections were then enzymatically digested for 75 minutes in papain at 2 mg/mL (Worthington Inc, Lakewood, NJ) at 37°C in an orbital shaker at a at 175 rpm. Tissue was then allowed to settle to 2 minutes, and papain solution was replaced with warm hibernate A (Invitrogen) B27 with glutamax (0.5 mM). Tissue was triturated 10 times for 45 seconds.

After allowing the tissue to settle, the upper 2 mL of supernatant was collected to a tube containing warm hibernate A B27 with glutamax (0.5 mM). The sediments were then resuspended and triturated again and supernatants were combined after four sets of trituration. The tissue was triturated in 8 mL of fresh hibernate A with glutamine

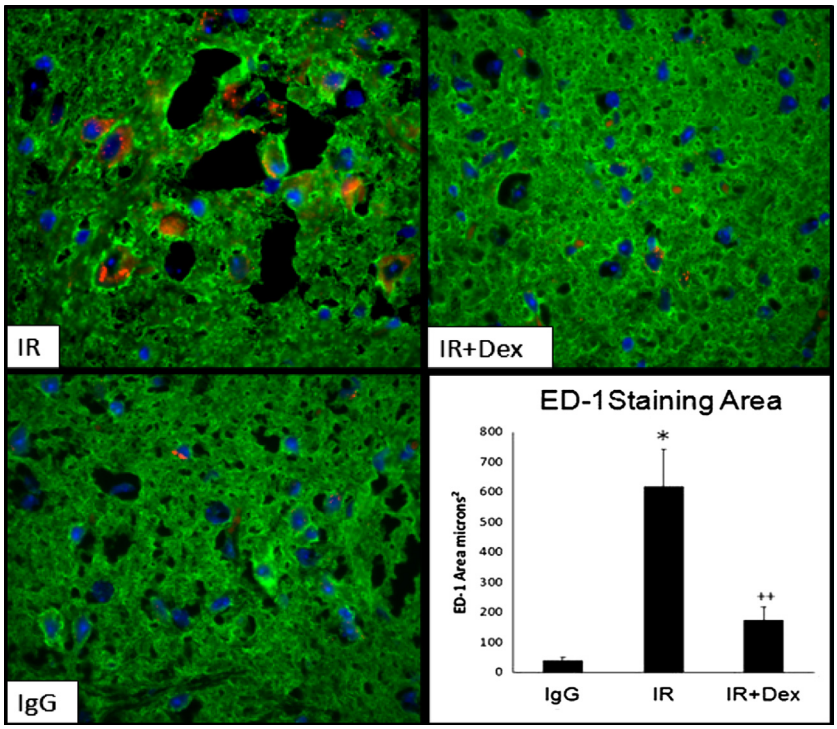

Fig 3. Microglia activation 12 hours following IR surgery was depicted by immunofluorescence of spinal cords incubation with anti-ED-1 on Cy3 channel (*red*) at ×40 magnification. Nuclei are resented by 4′,6-diamidino-2-phenylindole stain in *blue* with wheat germ agglutinin conjugated flourescein isothiocyanate staining in *green* for cytoplasm. All tissue incubated with anti-ED-1anti-body had a significant increase in the area of positive stain compared with IgG controls (*$P < .05$). Mice treated with dexmedetomidine (*IR+Dex*) had a significant reduction in ED-1 positive microglia compared with ischemia-reperfusion (*IR*) controls (++$P < .05$). Quantification for the area of tissue staining positive for anti-ED-1 antibody was performed and shown.

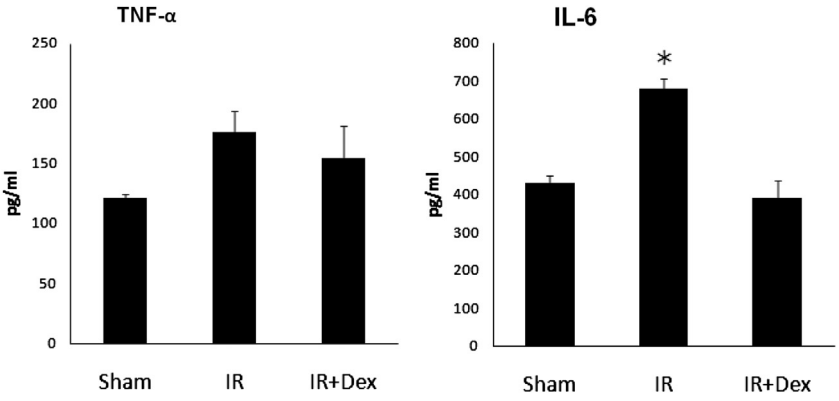

Fig 4. Concentrations of cytokines interleukin (*IL*)-6 and tumor necrosis factor (*TNF*)-α in spinal cord homogenates at 60 hours of reperfusion are represented. While TNF-α did a show slight decrease with dexmedetomidine treatment, it did not reach statistical significance. Treatment with dexmedetomidine (*IR+Dex*), however, did result in a significant in concentration of IL-6 compared with ischemia-reperfusion (*IR*) controls (*$P < .05$).

(0.5 mM) and B27. The cell suspension was layered over a 4-mL step gradient (Optipep diluted 0.505:0.495 [v/v]) with hibernate A−glutamine 0.5 mM−B27; 15%, 20%, 25%, and 35% (v/v) in hibernate A−glutamine 0.5 mM−B27 followed by centrifugation for 25 minutes, using 1800 rpm, at 24°C. The top 10 mL of the supernatant was aspirated. The bottom 1 mL was collected and diluted in 5 mL hibernate A−B27, and centrifugation was repeated at 1000 g for 5 minutes. The pellet was then resuspended in hibernate A−B27, and centrifuged again at 1000 g for

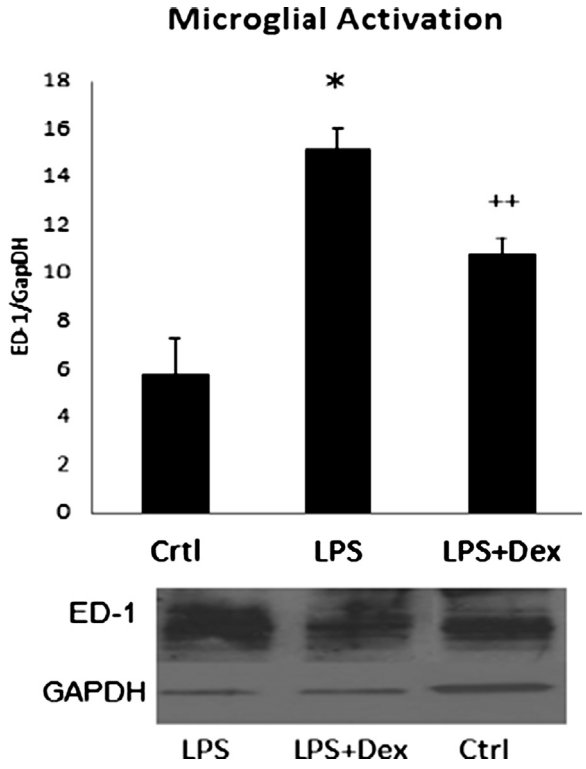

Fig 5. Following stimulation with lipopolysaccharide (*LPS*) microglia in both groups had a significant upregulation of activation marker ED-1 (*$P > .05$) compared with controls. Dexmedetomidine treatment (*LPS+Dex*) significantly reduced the expression of activation marker ED-1 compared with controls (*LPS*) (++$P < .05$).

5 minutes. The second pellet was resuspended in the culture medium with 20% fetal bovine serum, high-glucose Dulbecco's modified Eagle's medium (Invitrogen) with glutaGro and Na-pyruvate, penicillin-streptomycin then plated in poly-D-lysine coated wells.

LPS stimulation. Isolated cells were matured for at least 10 days to obtain confluence. Media was then replaced with serum free media for 12 hours followed by incubation with LPS *E coli 0111:B4* (InvivoGen, San Diego, Calif) 100 ng/mL based on previous studies.[14] Dexmedetomidine was added to media at a concentration of 10 mM prior to induction.[15] Media was collected following 24 hours of treatment for analysis of protein expression.

Cytokine quantification. Cytokines were quantified from spinal cord homogenate 60 hours following surgery or microglial media 24 hours following LPS stimulation using enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, Minn) performed according to the manufacturer's instructions. Absorbance of standards and samples were determined spectrophotometrically at 595 nm using a microplate reader (Bio-Rad, Hercules, Calif). Results were plotted against the linear portion of the standard curve, and the protein concentration of each sample was expressed as pg/mL of sample.

Immunoblotting. Microglia cells were lysed with M-PER mammalian protein extraction reagent (Fisher Scientific, Pittsburg, Pa). Protein extracts were placed in ×4 laemmli sample buffer with β-mercaptoethanol and boiled for 10 minutes at 100°C, loaded into a 10-well 4%-20% gradient ready gels (Bio-Rad), and run at 160 V for 50 minutes. The gels were transferred to nitrocellulose membranes at 100 V for 60 minutes, and then cross-linked using a UV Stratalinker (Stratagene, La Jolla, Calif). The membranes were blocked in 5% dry milk in 0.1% Tween in PBS (T-PBS). The blocked membranes were incubated with primary antibody overnight at 4°C (diluted to 1:1000 5% BSA in 0.1% T-PBS). Secondary incubated in appropriate horseradish peroxidase-conjugated antibodies diluted to 1:5000 in 5% BSA in 0.1% T-PBS then occurred for 1 hour at room temperature. SuperSignal West Dura chemiluminescent substrate (Thermo Scientific, Rockford, Ill) was used for 5 minutes. Membranes were visualized by Chemidoc Hi Sensitivity (Bio-Rad) scanner. Mean density values of bands were quantified by Quantity One software (Bio-Rad).

Statistical analysis. Statistical analysis was performed using analysis of variance with Fisher least significant difference post-hoc test (StatView; SAS Institute Inc, Cary, NC). Data are presented as mean ± standard error. A P value of $<.05$ was considered significant for all statistical comparisons.

RESULTS

Neurologic function. Hindlimb was function recorded using Basso Mouse Score (Fig 1). All mice that underwent IR surgery had some progressive, functional decline. Mice treated with dexmedetomidine (IR+Dex) did not have the same progression or severity as IR controls. A statistically significance was observed ($P < .05$) between treatment and control groups at 36 to 60 hours (Fig 1).

Histology and neuronal viability. Mice subjected to IR surgery that did not receive treatment (IR) exhibited poor gray-white matter differentiation and increased inflammatory infiltration compared with treated (IR+Dex) or sham mice (Fig 2). The quantity of normal appearing anterior horn neurons was also significantly decreased in all mice that underwent IR surgery compared with mice subjected only to sham surgery. Mice that received dexmedetomidine treatment postoperatively had significantly higher normal appearing anterior horn neurons compared with IR controls.

Spinal cord microglial activation. Spinal cords from mice 12 hours following IR surgery were assessed for microglial activation by ED-1, a specific marker of activation. Mice treated with dexmedetomidine at reperfusion had a significant reduction of ED-1 expression compared with mice in ischemic control groups (Fig 3).

Spinal cord cytokine expression. Spinal cord homogenate following IR or sham surgery was compared for cytokine production at 60 hours. Mice treated with dexmedetomidine (IR+Dex) had a significant reduction in the

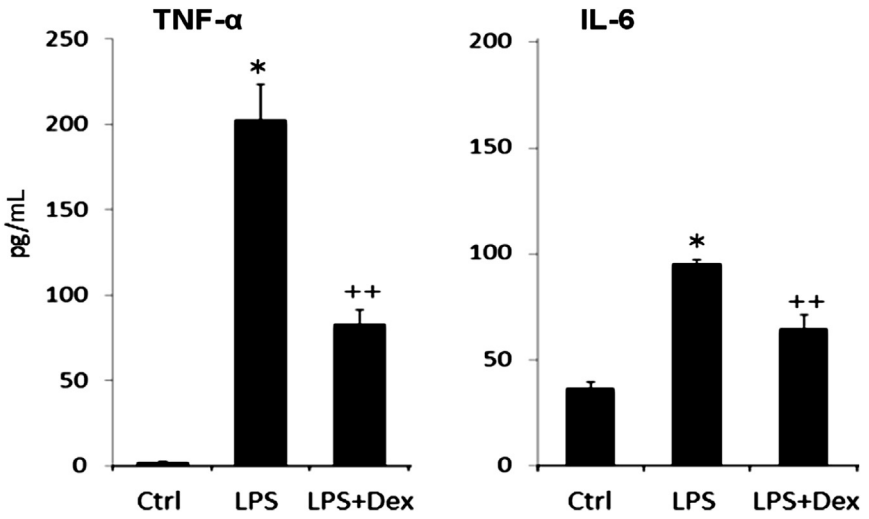

Fig 6. Microglial expression of cytokines tumor necrosis factor (*TNF*)-α and interleukin (*IL*)-6 were significantly increased following lipopolysaccharide (*LPS*) stimulation (*$P > .05$) compared with controls. Treatment with dexmedetomidine (*LPS+Dex*) significantly reduced the expression of both cytokines (++$P < .05$) compared with LPS treatment alone (*LPS*).

concentration of IL-6 compared with IR controls. TNF-α expression was reduced in treated mice compared with IR controls, however, this value did not reach statistical significance (Fig 4).

Microglial activation in isolation. Exposure of cells to LPS resulted in a significant increase in microglial activation compared with controls. Treatment with dexmedetomidine (LPS+Dex) resulted in a significant reduction in microglial activation after 24 hours of exposure to LPS (Fig 5).

Microglial cytokine production. Expression of the neurotoxic cytokines TNF-α and IL-6 was significantly elevated following exposure to LPS for 24 hours. Treatment of microglia with dexmedetomidine prior to LPS stimulation resulted in a significant attenuation of cytokine production (Fig 6).

CONCLUSIONS

Despite the evolution of surgical adjuncts and increased understanding of spinal cord ischemia-reperfusion injury, paraplegia remains a significant complication of thoracoabdominal aortic interventions. Patients with this injury exhibit either an immediate postoperative paralysis or develop a delayed injury following the surgery, with the latter becoming more prominent.[1] Delayed paraplegia, while equally as devastating, lends hope for the development of agents to reduce this complication. Investigation using our delayed paraplegia model[12] intends to create a pharmacologic adjunct to attenuation of this injury.

While immediate postoperative paraplegia directly correlates with duration of ischemia, delayed paraplegia from secondary injury appears to be more complex. Extrapolation of knowledge from stroke, ischemic, and traumatic models of spinal cord injury has given potential insight into these secondary neuroinflammatory sequelae and their contribution to progressive injury in the CNS.[16-18] In murine studies of loss of hindlimb motor function following thoracic aortic occlusion occurs in parallel with progressive production of inflammatory mediators.[19]

Our group has previously shown that combined treatment with dexmedetomidine before and after injury infers significant neuroprotection following temporary thoracic aortic occlusion.[20] While this neuroprotective effect in the spinal cord was consistent with those previous reported in the CNS[10,21] and other tissue beds[3,22] dexmedetomidine protective effect remains controversial.

Animal models and clinical trials have yielded considerable evidence that dexmedetomidine has significant anti-inflammatory properties. Rodent models of sepsis have shown than dexmedetomidine significantly reduces morality thought to be secondary to attenuation proinflammatory cytokine production.[23] Additionally, dexmedetomidine attenuated expression of proinflammatory markers in patients undergoing laparoscopic surgery.[24] Treatment was also associated with a reduction in length of ventilation and mortality in septic patients compared with standard sedation.[25]

In the current model of delayed paraplegia, even in the absence of preoperative treatment, dexmedetomidine was effective in preserving hindlimb function and neuronal viability. Furthermore, treatment was associated with an attenuation of microglial activation and cytokine production. Though these findings were not as significant as previously seen with pre- and postoperative administration, a protective effect was still evident.

While the reason for better protection with pretreatment remains unknown, it is likely multifactorial. One possibility is microglia activation prior to administration of dexmedetomidine could result in only a partial reduction

in injury. Dexmedetomidine has also been theorized to produce a host of other beneficial effects including modifications in neuronal ischemic tolerance[15] and reduced excitotoxicity.[26] The current findings are consistent with other models of spinal cord injury that have shown a reduction in proinflammatory cytokines following compressive injuries[27] and an attenuation of microglial activation in arthritic models.[28] However, these studies do not directly implicate dexmedetomidine's effect on microglia or subsequent neuroinflammation.

Microglia, the resident immune cells in the CNS, can produce a host of beneficial and toxic responses. Postinjurious microglial neurotoxicity has been shown by various models throughout the CNS. Systemic treatment with clodronate, an agent which depletes cells of macrophage lineage, protected the spinal cord from following traumatic injury.[29] Additionally, studies of cultured neuron and microglia have shown an amplification of neuronal injury when placed in co-cultures compared with isolation.[30]

It is now widely accepted that microglia can become activated into distinctly different phenotypes (denoted M1 and M2), which are either neurotoxic (M1) or neuroprotective (M2).[9] In the acute phase, the neurotoxic phenotype predominates.[31] Stimulation of microglial with LPS rapidly incites activation to the M1, neurotoxic, phenotype. While LPS is not the specific agonist in spinal cord injury following ischemia, it closely mimics other cellular signals such as heat shock proteins[32] or gangliosides[33] released from injured neurons.

To demonstrate the impact of dexmedetomidine on microglial signaling, spinal cord microglia were cultured in isolation and stimulated with LPS. Stimulation of microglia with LPS resulted in significant microglial activation and production cytokines, TNF-α, and IL-6. Treatment of microglia cultures with dexmedetomidine prior to LPS stimulation inferred a significant reduction in the expression of microglial activation marker ED-1 as well significant attenuation of cytokines.

Together this collection of data yields further evidence that the α-2 agonist dexmedetomidine exerts significant anti-inflammatory effects both in vivo and in vitro.

Furthermore, this reduction of microglial responses provides a potential mechanism for dexmedetomidine's neuroprotective effect and could one day be a potential target to reduce the incidence of paraplegia following thoracic aortic interventions.

AUTHOR CONTRIBUTIONS

Conception and design: MB, VA, FP, KF, PH, DF, JM, TR
Analysis and interpretation: MB, VA, FP, KF, PH, DF, JM, TR
Data collection: MB, VA, JM, KF, TR
Writing the article: MB, VA, FP, KF, JM, TR
Critical revision of the article: MB, VA, DF, PH, TR
Final approval of the article: MB, VA, FP, KF, PH, JM, DF, TR
Statistical analysis: MB, VA, KF, FP, TR
Obtained funding: FP, PH, DF, TR
Overall responsibility: MB

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